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1    **A Multi-Bacteriocin Cheese Starter System comprising Nisin and Lacticin 3147 in**  
2    ***Lactococcus lactis*, in Combination with Plantaricin from *Lactobacillus plantarum***

5    ***Running Title:* Multi-bacteriocin producing starter system**

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28 **ABSTRACT**

29 Functional starter cultures demonstrating superior technological and food safety  
30 properties are advantageous to the food fermentation industry. We evaluated the  
31 efficacy of single and double bacteriocin-producing starters of *Lactococcus lactis*  
32 capable of producing the Class I bacteriocins, nisin A and/or lacticin 3147 in terms of  
33 starter performance. Single producers were generated by mobilising the conjugative,  
34 bacteriophage resistance plasmid pMRC01, encoding lacticin genetic determinants, or  
35 the conjugative transposon Tn5276, encoding nisin genetic determinants, to the  
36 commercial starter *L. lactis* CSK2775. The effect of bacteriocin co-production was  
37 examined by superimposing pMRC01 into the newly constructed nisin transconjugant.  
38 Transconjugants were improved with regard to antimicrobial activity and bacteriophage  
39 insensitivity when compared to the recipient strain and the double producer was  
40 immune to both bacteriocins. Bacteriocin production in the starter was stable, although  
41 the recipient strain proved to be a more efficient acidifier than transconjugant  
42 derivatives. Overall, combining Class I bacteriocins (the double-producer or a  
43 combination of single producers) proved as effective as individual bacteriocins for  
44 controlling *Listeria innocua* growth in laboratory-scale cheeses. However, using the  
45 double producer in combination with the Class II bacteriocin producer *Lactobacillus*  
46 *plantarum*, or the lacticin producer with the Class II producer, proved most effective for  
47 reducing bacterial load. As emergence of bacteriocin tolerance was reduced 10-fold in  
48 the presence of nisin and lacticin, we suggest that the double producer in conjunction  
49 with the Class II producer could serve as a protective culture providing a food-grade,  
50 multi-hurdle approach to control pathogenic growth in a variety of industrial  
51 applications.

52

53 **IMPORTANCE**

54 We generated a suite of single and double-bacteriocin producing starter cultures capable  
55 of generating the Class I bacteriocins lacticin 3147 or nisin or both bacteriocins  
56 simultaneously via conjugation. The transconjugants exhibited improved bacteriophage  
57 resistance and antimicrobial activity. The single producers proved as effective as the  
58 double-bacteriocin producer at reducing *Listeria* numbers in laboratory-scale cheese.  
59 However, combining the double producer or the lacticin producing starter with a Class  
60 II bacteriocin producer, *Lactobacillus plantarum* LMG P-26358, proved most effective  
61 at reducing *Listeria* numbers, and was significantly better than a combination of the  
62 three bacteriocin producing strains, as the double producer is not inhibited by either of  
63 the Class I bacteriocins. Since the simultaneous use of lacticin and nisin should reduce  
64 the emergence of bacteriocin tolerant derivatives this study suggests that a protective  
65 starter system produced by bacteriocin stacking is a worthwhile multi-hurdle approach  
66 for food safety applications.

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## 78 INTRODUCTION

79 The development and characterisation of starter cultures that demonstrate superior  
80 technological properties such as improved proteolytic activity and flavour production,  
81 exopolysaccharide production or bacteriophage resistance are considered highly  
82 advantageous within the food fermentation industry (1). The ability of starter strains to  
83 produce bacteriocins is also considered an important technological trait for controlling  
84 undesirable and/or pathogenic growth in situ and for improving sensory characteristics  
85 (1-3). Bacteriocins are ribosomally synthesised, heat stable antimicrobial peptides that  
86 generally act by depolarising the target cell membrane and/or through inhibiting cell  
87 wall synthesis where the producing strain is immune to the antimicrobial effect (4).  
88 They comprise a highly heterogeneous group that have recently been divided into three  
89 distinct Classes (5).

90 The exploitation of bacteriocin-producing cultures is a particularly attractive  
91 option for the food industry owing to the generally recognized as safe (GRAS) status of  
92 the cultures, immediately fulfilling the consumers' demand for minimally processed  
93 foods lacking artificial food additives. The bacteriocin producer can serve as the starter  
94 culture or be added as an additional protective culture. Several studies have highlighted  
95 the efficacy of such approaches where bacteriocin-producing cultures have proven  
96 effective for inhibiting the growth and proliferation of pathogenic and food spoilage  
97 microorganisms (6-9). Despite this, the use of bacteriocins in the food industry remains  
98 limited possibly owing to the fact that a bacteriocin alone may not be capable of  
99 providing sufficient protection against contamination (10). The use of bacteriocin  
100 combinations or bacteriocin stacking may represent an alternative approach. Indeed,  
101 improved antimicrobial activity of bacteriocin combinations has been reported  
102 previously (11, 12, 13). However, when using multiple bacteriocins, it is essential that

103 other important cultures are not inhibited. This can be overcome to some degree by  
104 developing a multi-bacteriocinogenic culture which is immune to the bacteriocins it  
105 produces.

106 In the present study, we generated single and double bacteriocin-producing  
107 cultures of *L. lactis* CSK2775 with the capacity to produce Class I bacteriocins, lacticin  
108 3147 (hereafter lacticin), nisin A (hereafter nisin) or lacticin and nisin. Both  
109 bacteriocins target lipid II to generate pores in the cell membrane causing proton-  
110 motive force dissipation and subsequent cell death (14-17). Resulting transconjugants  
111 were assessed for bacteriocin production, bacteriophage resistance properties,  
112 acidification efficiency and antimicrobial activity against a spectrum of indicator strains  
113 including food pathogens and other lactic acid bacteria (LAB). The ability of the  
114 transconjugants (single and double) to produce bacteriocins in laboratory-scale cheese  
115 was assessed and we also evaluated the anti-listerial potential of the Class I producers  
116 alone and in combination with the Class IIa bacteriocin producer *Lactobacillus*  
117 *plantarum* LMG P-26358 (18). Class IIa bacteriocins cause pore formation by binding  
118 to and irreversibly opening the sugar transporter mannose phosphotransferase (Man-  
119 PTS) system in the target cell (19). In this study, *Listeria innocua* served as a surrogate  
120 for *Listeria monocytogenes* for reasons of safety and efficiency (as in many other  
121 studies) and because *L. innocua* has been successfully used in previous studies  
122 investigating the anti-listerial potential of nisin (20-24), lacticin (25-28) and plantaricin  
123 (18) in food systems.

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## 128 MATERIALS AND METHODS

### 129 Bacterial strains and media

130 Bacterial strains used in this study are listed in Tables 1 and 2. *L. lactis* strains were  
131 routinely propagated at 30°C in M17 medium (Difco Laboratories, Detroit, MI, USA)  
132 supplemented with 0.5% (w/v) lactose (LM17) or glucose (GM17). *Lb. plantarum* was  
133 grown in MRS medium (29) (Difco Laboratories) at 30°C. *L. innocua* was routinely  
134 propagated in GM17 broth at 37°C containing 500 µg/ml streptomycin (Sigma Aldrich,  
135 Ireland). Other media used in this study include BHI (Brain-Heart Infusion) broth  
136 (Oxoid Ltd., Basingstoke, Hampshire, England) and RCM (Re-inforced Clostridial  
137 Medium) (Merck, Darmstadt, Germany). All strains were stored in 50% glycerol at -  
138 20°C.

139

### 140 Strain construction and analytical tests

#### 141 Strain construction

142 The conjugation method of Coakley et al. (30) was used with slight modifications to  
143 generate lacticin transconjugants. Inocula (2%) of both donor and recipient were grown  
144 for 4 h in GM17 broth at 30°C. After the growth period, 1 ml of recipient and 1 ml of  
145 donor were harvested by centrifugation (16,000 x g for 1 min) and rinsed twice with  
146 GM17 broth. After the final rinse, each strain was resuspended in 50 µl of GM17 broth.  
147 The concentrated recipient and donor (20x) were then mixed with each other at the  
148 following ratios, 1:1, 2:1 and 20:1. Each mixture was spotted onto the centre of a GM17  
149 agar plate and incubated for 18 h at 30°C. The following day, spots were harvested in 1  
150 ml of maximum recovery diluent (MRD; Oxoid) and serially diluted before plating on  
151 lactose indicator agar (LIA) containing lacticin (400 arbitrary units (AU)/ml) as  
152 described previously (30). Following 48 h of incubation at 30°C the lacticin-containing

LIA plates were examined for lactose-positive colonies (yellow) against a background of lactose-negative colonies (white), and lactose-positive colonies were selected and grown in LM17 broth for further analysis.

Nisin transconjugants were generated according to the method of Gireesh et al. (31) with modifications: inocula of donor (1.5%) and recipient (2%) were grown for 4 h in GM17 broth at 30°C. Donor and recipient were then mixed at the following ratios, 1:10 and 1:100 in the presence of 400 µg/ml α-chymotrypsin (Sigma Aldrich). The cells were collected onto membrane filters (0.45 µm pore size, Merck, Millipore, Darmstadt, Germany) after which the filters were placed on GM17 agar plates (cell side down). Following 18 h of incubation at 30°C, cells were harvested from the filter and added to 10% reconstituted skimmed milk (RSM) containing 400 AU/ml nisin (Sigma Aldrich) and incubated at 30°C for 24-48 h. Clotted samples were serially diluted, plated on LIA and following 48 h of incubation at 30°C, yellow colonies were selected for further analysis.

167

#### *Bacteriocin production and immunity*

Bacteriocin production and immunity was assessed by performing the agar well diffusion assay as described by Ryan et al. (32). Indicator organisms are listed in Table 2. Bacteriocin sensitivity was scored according to the diameter of the zone of inhibition surrounding the well which contained cell free supernatant from the bacteriocin producer. The concentration of bacteriocin produced by the double producer was measured by agar well diffusion assay using a serial two-fold dilution of the filtered culture supernatant and bacteriocin activity was calculated as the inverse of the last dilution that gave a definite zone of clearance after overnight incubation where AU were expressed per ml.



178 *Colony mass spectrometry*

179 Colony mass spectrometry was performed according to the method described by Field  
180 et al. (33).

181

182 *PCR scan*

183 Genomic DNA was extracted from 1.5 ml of 18 h cultures according to the method of  
184 Hoffman and Winston (34) slightly modified as described previously (35). Primer pairs  
185 used to scan strains for the presence of pMRC01 as well as the genes associated with  
186 nisin production are listed in Table 3. PCR was performed in a Hybaid PCR express  
187 unit (Hybaid Ltd., Middlesex, UK) using MyTaq<sup>TM</sup> Red Mix polymerase (Bioline Ltd.,  
188 London, U.K.) according to manufacturers' specifications combined with an annealing  
189 temperature of 55°C.

190

191 *Pulsed field gel electrophoresis*

192 Pulsed field gel electrophoresis (PFGE) was performed according to Mills et al. (35)  
193 using the restriction enzyme SmaI (New England Biolabs, Hertfordshire, U.K.). DNA  
194 fragments were run on a CHEF-DR III pulsed-field system (Bio-Rad laboratories,  
195 California, USA) at 6V/cm for 22 h with a 1-30 s linear ramp pulse time. Molecular size  
196 markers (N0340S, N0350S) were purchased from New England BioLabs.

197

198 *Bacteriophage assays*

199 Bacteriophages were propagated according to the method outlined previously (36).  
200 Sensitivity to bacteriophage infection was performed by the double agar layer plaque  
201 assay as described previously (30).

202

203 *Characterisation of acid production*

204 Acid production was monitored in 10% RSM in the presence and absence of 0.1% yeast  
205 extract according to the method of Harrington and Hill (37).

206

207 **Laboratory-scale cheese manufacture**

208 Cultures were grown from frozen stocks in their respective media for 18 h (Table 1).

209 The cultures were then inoculated at 1% (v/v) into 10% (w/v) RSM and incubated for a  
210 further 18 h at 30°C. In the case of *Lb. plantarum* LMG P-26358, the culture was grown  
211 in 10% RSM containing 0.1% (v/v) yeast extract and 0.2 g/l MnSO<sub>4</sub>·4H<sub>2</sub>O as previously  
212 reported (18).

213 One litre vats of whole milk heated to 31°C were inoculated with the 18 h RSM cultures  
214 as follows:

215 -Vat 1 = 0.75% (v/v) *L. lactis* DPC4268, 0.75% (v/v) *L. lactis* CSK2775 (no  
216 bacteriocin)

217 -Vat 2 = 0.75% (v/v) *L. lactis* DPC4268, 0.75% (v/v) *L. lactis* CSK3281 (nisin  
218 producer)

219 -Vat 3 = 0.75% (v/v) *L. lactis* DPC4268, 0.75% (v/v) *L. lactis* CSK3594 (lacticin  
220 producer)

221 -Vat 4 = 0.75% (v/v) *L. lactis* DPC4268, 0.75% (v/v) *L. lactis* CSK3533 (nisin-lacticin  
222 double producer)

223 -Vat 5 = 0.75% (v/v) *L. lactis* DPC4268, 0.5% (v/v) *L. lactis* CSK3281 (nisin  
224 producer), 0.5% (v/v) *Lb. plantarum* LMG P-26358 (plantaricin producer)

225 -Vat 6 = 0.75% (v/v) *L. lactis* DPC4268, 0.5% (v/v) *L. lactis* CSK3594 (lacticin  
226 producer), 0.5% (v/v) *Lb. plantarum* LMG P-26358 (plantaricin producer)

227 -Vat 7 = 0.75% (v/v) *L. lactis* DPC4268, 0.5% (v/v) *L. lactis* CSK3533 (nisin–lactacin  
228 double producer), 0.5% (v/v) *Lb. plantarum* LMG P-26358 (plantaricin producer)  
229 -Vat 8 = 0.75% (v/v) *L. lactis* DPC4268, 0.5% (v/v) CSK3594 (lactacin producer), 0.5%  
230 (v/v) CSK3281 (nisin producer)  
231 -Vat 9 = 0.75% (v/v) *L. lactis* DPC4268, 0.5% (v/v) CSK3594 (lactacin producer), 0.5%  
232 (v/v) CSK3281 (nisin producer), 0.5% (v/v) *Lb. plantarum* LMG P-26358 (plantaricin  
233 producer)  
234 A streptomycin resistant derivative of *L. innocua* (DPC6578) grown for 18 h was added  
235 to each vat at a level of  $10^4$  cfu/ml. Thirty min after inoculation, 150 international milk  
236 clotting units/ml Kalase rennet (CSK Food Enrichment, The Netherlands) was added  
237 according to manufacturer's specifications and after a further 15 min the curd was cut  
238 into cubes. Following a 10 min stirring step, approximately 35% of the whey was  
239 removed and the curd was stirred for a further 5 min. The temperature was then  
240 elevated to 36°C over a 5 min period and the curd was stirred for a further 20 min. The  
241 curd was further drained and lightly pressed into moulds for 20 min before pressing  
242 overnight. After 24 h the cheeses were submerged in a brine bath (23% NaCl [w/v],  
243 0.22% phosphoric acid [v/v], 0.1% NaOH [w/v], 0.6% CaCl<sub>2</sub> [w/v]) at 10–12°C for 5 h  
244 after which they were vacuum-packed and ripened at 7°C for 4 weeks. *L. innocua*  
245 DPC6578 was enumerated in each cheese on a weekly basis by homogenising 1 g of  
246 cheese in 2% sterile tri-sodium citrate and plating serial dilutions on selective medium  
247 (GM17 agar with 500 µg/ml of streptomycin). The cheese trial was performed in  
248 triplicate and sampling for each trial was performed in duplicate.  
249 Nisin ( $3352 \pm 3$  Da), lactacin (Ltnβ:  $2847 \pm 4$  Da) and plantaricin ( $3928 \pm 3$  Da) present  
250 within cheese samples from Vats 4, 5, 6, and 7 were verified by MALDI-TOF mass  
251 spectrometry (MALDI-TOF MS) as described previously (18). In the case of lactacin,

the presence of the correct mass for Ltn $\beta$  was indicative of lactacin since Ltn $\alpha$  can be difficult to detect in a complex fraction. All fractions were tested for antimicrobial activity by agar well diffusion assays against the appropriate indicator strains (lactacin and nisin against *L. lactis* HP; plantaricin against *L. innocua*) where mass and concomitant activity were indicative of bacteriocin presence. Fractions expected to contain the lactacin peptides (23/24: Ltn $\alpha$  and 37: Ltn $\beta$ ) were combined or wells were positioned near each other to assess lactacin activity.

259

#### 260 **Frequency of bacteriocin resistance/tolerance**

To determine the frequency of bacteriocin resistance/tolerance development in *L. innocua*, freshly prepared 18 h cultures were serially diluted in MRD and spread plated on to GM17 or GM17 containing either 1000 AU/ml or 320 AU/ml of the appropriate bacteriocin or bacteriocin combination, the latter concentration representing the arbitrary in situ concentration of the bacteriocins in the cheeses. Plates were incubated aerobically at 37°C for up to 48h, at which time, the frequency of bacteriocin resistance/tolerance was calculated as described previously (38). All experiments were performed in triplicate.

269

#### 270 **Statistical Analysis**

*Listeria* counts in laboratory-scale cheeses were statistically analyzed using one-way ANOVA. Post hoc multiple comparisons were determined by Tukey's test and differences were considered to be statistically significant at  $P < 0.05$ . Statistical tests were performed using XLSTAT statistical software.

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## 277 RESULTS

### 278 *Transconjugant validation*

279 The presence of the plasmid, pMRC01, and the nisin transposon, Tn5276, in *L. lactis*  
280 CSK2775 transconjugants was validated by PCR using plasmid- and transposon-  
281 specific primers, respectively (Fig. 1). PCR analysis confirmed that the genetic  
282 determinants responsible for lacticin production were present in 2775 (pMRC01)  
283 creating the lacticin transconjugant *L. lactis* CSK3594, and confirmed the presence of  
284 the nisin genetic determinants in 2775 (Tn5276) creating the nisin transconjugant  
285 CSK3281. The presence of the lacticin and nisin genetic determinants was confirmed in  
286 the double producer resulting in the nisin-lacticin transconjugant *L. lactis* CSK3533. To  
287 confirm the identity of each transconjugant, genomic fingerprints were generated by  
288 PFGE with the restriction endonuclease, SmaI. All transconjugants analysed generated  
289 the same restriction pattern as the recipient strain, CSK2775 (results not shown). Well  
290 diffusion assays confirmed that CSK3594 was sensitive to nisin and that CSK3281 was  
291 sensitive to lacticin but the double producer was immune to both bacteriocins.

292 Colony mass spectrometry (CMS) confirmed that CSK3594 and CSK3533 each  
293 produced a peptide with a mass of approximately  $2847 \pm 4$  Da corresponding to the  
294 lacticin peptide, Ltn $\beta$  (Fig. 2). However, lacticin peptides (Ltn $\alpha$  or Ltn $\beta$ ) could not be  
295 detected in the recipient strain, CSK2775 (Fig. 2). CMS also detected a peptide with a  
296 mass of  $3352 \pm 3$  Da corresponding to nisin in strains CSK3281 and CSK3533; this  
297 peptide was absent in the recipient strain. These data confirm that lacticin is produced  
298 by CSK3594, nisin is produced by CSK3281, and that both nisin and lacticin are  
299 produced by CSK3533 (Fig. 2).

300 The level of inhibitory activity in the culture supernatant of the double producer,  
301 CSK3533, against *L. lactis* HP was determined to be 1000 AU/ml when measured by

302 agar well diffusion assays, corresponding to a zone size of 4.5 mm which is equivalent  
303 to the zone size produced by the nisin transconjugant *L. lactis* CSK3281. The lacticin  
304 transconjugant, CSK3594 produced a 2.5 mm zone against *L. lactis* HP. To our  
305 knowledge, this is the first report of the successful construction of a food-grade  
306 commercial *L. lactis* starter strain capable of producing both nisin and lacticin 3147,  
307 two potent Class I bacteriocins.

308

#### 309 *Strain performance and stability*

310 The stability of the bacteriocin/lactose positive phenotype in each transconjugant was  
311 confirmed via repeated “passaging” in GM17 followed by bacteriocin activity assays  
312 against the indicator, *L. lactis* HP. Bacteriocin production and immunity in CSK3281,  
313 CSK3594 and CSK3533 proved to be stable over time. However, upon passaging of the  
314 double producer, CSK3533, in GM17, a mixed culture containing lactose fermenting  
315 and non-fermenting colonies could be observed when plated on LIA. This mixed culture  
316 was subsequently attributed to the loss of a large plasmid (>50 kb) present in CSK3533  
317 (confirmed by plasmid profile analysis; results not shown) and is presumed to be  
318 involved in lactose metabolism. The lactose fermenting phenotype could be preserved  
319 through the supplementation of lactose to the growth medium.

320 Comparative analyses of acidification profiles of each transconjugant and the  
321 recipient strain revealed that the bacteriocin-free recipient, CSK2775, proved to be the  
322 most efficient acidifier (Fig. 3). Although the lacticin single producer (CSK3594) was  
323 more efficient than the nisin single producer (CSK3281), both proved to be more  
324 efficient than the double producer, CSK3533 (Fig. 3). The addition of 0.1% yeast  
325 extract improved lactic acid production in the transconjugants.

326

327 *Spectrum of inhibition and bacteriophage resistance*

328 The activity of the single bacteriocin producers CSK3594 and CSK3281 as well as the  
329 double producer, CSK3533, were assayed against a range of indicator strains including  
330 food spoilage, pathogenic bacteria, as well as LAB, and non starter LAB (NSLAB)  
331 (Table 2). The single lacticin producer was found to inhibit primarily lactococci,  
332 lactobacilli, and clostridia while a wider spectrum of inhibition was observed for both  
333 the nisin producer and the double producer. The double producer proved to be more  
334 effective than either lacticin or nisin single producers with regard to *Clostridium*  
335 *tyrobutyricum* inhibition producing a 6 mm zone while the lacticin producer, CSK3594,  
336 and nisin producer, CSK3281, each produced zones of 4 mm and 3 mm, respectively.  
337 Interestingly, the recipient strain, CSK2775, also produced a 1 mm zone against  
338 *Clostridium tyrobutyricum* suggesting that some other antimicrobial effect is potentially  
339 working in conjunction with the bacteriocins in the transconjugants. In addition, the  
340 double producer generated a 4 mm zone against CSK3281 in comparison to a 2.5 mm  
341 zone produced by CSK3594. This increase in zone size is surprising given that  
342 CSK3281 harbours the genetic machinery for nisin immunity and indeed was proven to  
343 be immune to nisin in the antimicrobial assays. However, the increased susceptibility of  
344 the nisin transconjugant to lacticin may be due to a lower cell density in the seeded  
345 plate as a consequence of a slower growth rate although this has not been confirmed.

346 Bacteriophage sensitivity assays confirmed that CSK2775, the non-  
347 bacteriocinogenic recipient, was sensitive to all bacteriophages analysed (Table 4). The  
348 nisin producer, CSK3281 was resistant to 50% of the bacteriophages analysed, while  
349 the lacticin single producer and the double producer were each resistant to 80% of  
350 bacteriophages analysed (Table 4).

351

352 *Laboratory-scale cheese production*

353 To analyse the in situ inhibitory activity of the bacteriocin producers (single, double and  
354 in combination with the plantaricin producer, *Lb. plantarum* LMG P-26358) laboratory-  
355 scale cheeses were manufactured with the fast acidifier *L. lactis* DPC4268 and the  
356 bacteriocin producers served as protective cultures. The cheeses were spiked with  $10^4$   
357 cfu/ml of *L. innocua*. Each cheese was ripened for 4 weeks at 7°C; *Listeria* was  
358 enumerated weekly during the ripening period. Fig. 4 shows *Listeria* viable cell counts  
359 over the 4 week period where bacteriocin containing vats were compared with Vat 1 (no  
360 bacteriocin) at each week. At week 0, *Listeria* numbers were significantly different  
361 between Vat 1 (5.6 log cfu/g) and all other vats with lowest *Listeria* numbers recorded  
362 for vat 7 (CSK3533; *Lb. plantarum*) at 3.5 log cfu/g ( $P<0.001$ ), followed by Vat 2  
363 (CSK3281) at 3.8 log cfu/g ( $P<0.001$ ). *Listeria* numbers in the remaining vats were  
364 reduced by 0.9 to 1.5 logs when compared to Vat 1. By week 1, *Listeria* numbers in Vat  
365 7 continued to decrease significantly compared to Vat 1 with a 3 log reduction recorded  
366 ( $P<0.001$ ). *Listeria* numbers in Vat 6 (CSK3594; *Lb. plantarum*) were also significantly  
367 different to Vat 1 with a 2.7 log reduction ( $P<0.001$ ). Significant reductions were also  
368 observed for Vat 9 (CSK3281; CSK3594; *Lb. plantarum*) (1.6 log reduction;  $P<0.01$ )  
369 and Vat 5 (CSK3281; *Lb. plantarum*) (0.8 log reduction;  $P<0.05$ ). At week 2 lowest  
370 *Listeria* numbers were recorded for Vat 6 (0.4 log cfu/g) and Vat 7 (0.7 log cfu/g)  
371 representing 2.6 and 2.3 log reductions compared to Vat 1 (3 log cfu/g) ( $P<0.01$ ). Vat 5  
372 (CSK3281; *Lb. plantarum*) was also significantly different to Vat 1 (1.8 log reduction;  
373  $P<0.01$ ). By week 3, Vat 7 exhibited lowest *Listeria* numbers (0.4 log cfu/g) followed  
374 by Vat 9 (0.8 log cfu/g), which were both significantly different to Vat 1 (2.5 log cfu/g)  
375 ( $P<0.05$ ). By the week 4, *Listeria* numbers for Vat 1 (2.9 log cfu/g) were significantly  
376 different to most other Vats, with no *Listeria* detected in Vat 6 ( $P<0.01$ ) and numbers



377 reduced to 0.3 log cfu/g for Vat 7 ( $P<0.01$ ). *Listeria* numbers in the remaining vats (2,  
378 3, 4, 8, 9) ranged from 0.8 to 1 log cfu/g representing significant differences compared  
379 to Vat 1 ( $P<0.05$ ). While, *Listeria* numbers in Vat 5 were not deemed significantly  
380 different to Vat 1, they approached a significant reduction ( $P=0.07$ ).

381 In terms of the lacticin and nisin transconjugants (without *Lb. plantarum*), no  
382 significant differences were observed between the double producer (Vat 4) and either of  
383 the single producers (Vats 2 and 3) with regards to *Listeria* numbers at any week. We  
384 then compared Vat 8 which consists of the two single producers (CSK3281; CSK3594)  
385 (4.4 log cfu/g; week 0) with Vats 2, 3 and 4. While Vat 8 was found to be significantly  
386 different to Vat 2 (3.7 log cfu/g) at week 0 ( $P<0.05$ ) whereby the nisin producer  
387 generated greater *Listeria* reductions than the combination of nisin and lacticin single  
388 producers, no significant differences were observed for weeks 1-4. Likewise, no  
389 significant differences were observed between Vat 8 and Vats 3 or 4 over the ripening  
390 period.

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#### 392 *Bacteriocin detection in Vats 4, 5, 6 and 7*

393 The correct masses for nisin (fraction 21), Ltn $\beta$  (fraction 37) and plantaricin (fraction  
394 19) were detected by MALDI-TOF MS in Vat 7 (double producer and plantaricin) at  
395 week 0 (Fig 5A). Antimicrobial assays also revealed that these fractions contained  
396 activity (lacticin activity was restored by combining fractions 23 (Ltn $\alpha$ ) and 37).

397 At week 4, a mass corresponding to plantaricin was detected in fraction 19 but there  
398 was no antimicrobial activity. Nisin was detected in fraction 21 at week 4 and activity  
399 was also confirmed. The correct mass for Ltn $\beta$  could not be detected at week 4. Despite  
400 this, combining fractions 23 and 37 did yield a zone of inhibition against the indicator  
401 strain, suggesting the bacteriocin is present.

402 The correct mass for nisin was not detected in Vat 4 (double producer) at weeks  
403 0 or 4, however, fraction 21, which is generally expected to contain nisin, yielded a  
404 zone of inhibition against the indicator strain on both weeks, suggesting the bacteriocin  
405 is present (Fig 5B). The correct mass for Ltn $\beta$  was detected in Vat 4 (double producer)  
406 at weeks 0 and 4 (Fig 5B). Lacticin activity was confirmed when fractions 23 and 37  
407 were positioned beside each other in the agar well diffusion assays.

408 Vat 5 (nisin and plantaricin) was found to contain the nisin mass at weeks 0 and  
409 4 although the correct mass was found in fraction 22 at week 0 and in fraction 23 at  
410 week 4 (Fig 5C). This is presumably due to slight variations in the times the peptide  
411 eluted from the HPLC. These fractions exhibited antimicrobial activity against the  
412 indicator strain but the zones were smaller than previously observed. The plantaricin  
413 mass was not detected in Vat 5 at week 0 but a zone of inhibition against *Listeria* was  
414 observed for fraction 19. However, the plantaricin mass was detected in Vat 5 at week 4  
415 and antimicrobial activity was confirmed.

416 Vat 6 (lacticin and plantaricin) was shown to contain the correct Ltn $\beta$  and plantaricin  
417 masses at weeks 0 and 4 in fractions 37 and 19, respectively (Fig 5D). Antimicrobial  
418 activity was confirmed for fraction 19 although the zone was smaller at week 0 when  
419 compared to week 4. In the case of lacticin, combining fractions 24 (Ltn $\alpha$ ) and 37  
420 confirmed antimicrobial activity.

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#### 422 *Development of bacteriocin tolerance*

423 The frequency of tolerance/resistance development in *L. innocua* was assessed using  
424 1000 AU/ml of each bacteriocin. The frequency of resistance against 1000 AU/ml of  
425 nisin was calculated to be  $6.56 \times 10^{-4}$ . Resistance development could not be observed  
426 when *Listeria* was exposed to 1000 AU/ml lacticin or 1000 AU/ml nisin and lacticin.

427 On the other hand, the frequency of resistance development against 320 AU/ml nisin or  
428 320 AU/ml lactacin (representing arbitrary concentrations in cheese) was much lower at  
429  $4.9 \times 10^{-1}$  and  $3.02 \times 10^{-1}$ , respectively. Simultaneous exposure to lactacin and nisin at  
430 320 AU/ml decreased the frequency of resistance to  $3.18 \times 10^{-2}$ . However, bacteriocin  
431 resistant colonies remained sensitive to 1000 AU/ml indicating that *Listeria* cells were  
432 tolerant rather than completely resistant.

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452 **DISCUSSION**

453 In this study, an isogenic family of nisin and lactacin transconjugants was developed  
454 with a view to better understand the impact of multiple bacteriocin production and  
455 genetic load on starter culture functionality. Genotypic and phenotypic analyses  
456 including PCR, well diffusion assays and CMS confirmed the acquisition of lactacin  
457 and/or nisin in each transconjugant.

458 In agreement with previous findings (39) which revealed that pMRC01 imposes  
459 a burden on lactococcal metabolism affecting growth and acidification rates, the  
460 presence of pMRC01 was shown to influence lactococcal acidification in the lactacin  
461 transconjugant, as did the presence of the nisin transposon in the nisin transconjugant.  
462 However, bacteriocin stacking resulted in slowest acidification rates but which can be  
463 overcome with the addition of 0.1% yeast extract to the double producer. Yeast extract  
464 presumably lessens the burden of plasmid and transposon acquisition as it provides  
465 amino acids along with purine and pyrimidine bases and inorganic constituents which  
466 have been shown to stimulate lactococcal growth (40). We therefore suggest that the  
467 double producer has potential to serve as a protective culture when used in conjunction  
468 with a suitable acidifier. Despite this, the lactose utilization phenotype in the double  
469 producer, CSK3533, was found to be unstable in GM17 apparently due to the loss of a  
470 large (>50kb) plasmid likely involved in lactose utilization. Both the plasmid instability  
471 and slower acidification profiles observed in the double producer (CSK3533) may be  
472 attributed to the metabolic burden imposed by the presence of pMRC01 and the nisin  
473 transposon. In an effort to ease the metabolic load, it is possible that as the energy  
474 demand of the cell increases and metabolites are exhausted, a reduction in growth rate  
475 and perhaps the loss of nonessential plasmids may occur (39, 41). This is supported by

476 the fact that the addition of lactose to M17 broth maintains the lactose utilization  
477 phenotype in the double producer.

478 Antimicrobial activity assays confirmed that co-production of nisin and lactacin  
479 by CSK3533 was as effective as its single producing counterparts against most of the  
480 indicator strains tested. This indicates that bacteriocin production is not affected by the  
481 slower growth rate observed in the double producer. Furthermore, with the exception of  
482 *C. tyrobutyricum*, the co-production of the two potent bacteriocins did not result in an  
483 increase in antimicrobial activity. Studies suggest that bacteriocin production and  
484 subsequent inhibition may be influenced by growth medium (42, 43). The multi-  
485 bacteriocinogenic strain *L. lactis* INIA 415 was capable of producing the Class I  
486 bacteriocin lactacin 481 in M17 broth only and produced nisin in milk only (42).  
487 Therefore, it is possible that growth of CSK3533 under different conditions could result  
488 in higher levels of nisin or lactacin being produced.

489 In terms of bacteriophage resistance, transconjugants were shown to be more  
490 resistant to bacteriophage attack than the recipient strain with superior resistance  
491 properties observed in pMRC01 derivatives, which is known to harbour an abortive  
492 infection mechanism (30).

493 Laboratory-scale cheese inoculated with *Listeria* was used to assess the efficacy  
494 of single and double bacteriocin producers alone and in combination with the  
495 plantaricin producer, *Lb. plantarum* LMG P-26358 in situ. The latter strain was  
496 previously shown to have a narrow spectrum of inhibition, inhibiting *Listeria* and  
497 enterococcal strains but not clostridia, *E. coli*, *Bacillus* species, *Salmonella*, or members  
498 of the LAB (18). The strain proved to be an effective adjunct for controlling *Listeria*  
499 growth in a cheese model (18). In the present study, CSK3594, CSK3281 and CSK3533  
500 failed to inhibit *L. innocua* by agar well diffusion assay, however, by the end of the

501 ripening period, *Listeria* numbers from cheeses prepared with these starters were  
502 significantly reduced when compared to the control, Vat 1 (no bacteriocin).  
503 Interestingly, the double producer combined with the plantaricin producer exhibited the  
504 greatest reduction in *Listeria* numbers at week 0, a trend which continued to Week 1  
505 suggesting the effectiveness of this combination for reducing initial bacterial load. The  
506 inhibitory effect of this combination was on the whole significantly better than using  
507 both single producers with the plantaricin producer. This can be explained by the fact  
508 that the nisin producing transconjugant inhibits the lacticin transconjugant and vice  
509 versa whereas the double producer is immune to both bacteriocins. The combination of  
510 the lacticin producer with *Lb. plantarum* LMG P-26358 also significantly reduced  
511 *Listeria* numbers by week 1 and indeed by week 4, *Listeria* could not be detected in this  
512 vat. Overall, the double producer combined with the plantaricin producer followed by  
513 the lacticin producer combined with plantaricin exhibited the most significant  
514 reductions in *Listeria* numbers over the ripening period.

515 In general, a similar inhibitory trend was observed amongst the single Class I  
516 producers, the double producer or the combined single producers which were  
517 significantly different to Vat 1 at week 0 and week 4. While the double producer did not  
518 alter *Listeria* numbers significantly when compared to the single producers alone, a 10-  
519 fold reduction in the emergence of bacteriocin tolerance was observed when *Listeria*  
520 was exposed to both nisin and lacticin, suggesting that bacteriocin stacking could be an  
521 effective method to prevent pathogen growth in food applications. However, combining  
522 bacteriocins from different Classes or sub-classes is considered most effective for  
523 reducing the emergence of resistance (44) which explains the increased antimicrobial  
524 efficacy for vats containing the Class I and Class II bacteriocins in this study.

525 Interestingly, cheese prepared with non-bacteriocinogenic CSK2775 also  
526 resulted in reduced *Listeria* numbers over the four week period although to a lesser  
527 extent than the bacteriocin containing cheeses. Therefore, it is probable that bacterial  
528 competition coupled with unfavourable conditions relating to cheese manufacture  
529 including lactic acid and high salt concentrations have provided a hurdle-effect to cause  
530 the observed reductions. Indeed, several intrinsic factors including moisture content,  
531 acidity and competitive flora are known to dictate pathogen survival in cheese (45, 46).

532 MALDI-TOF MS of cheeses from vats 4, 5, 6 and 7 indicated that nisin and  
533 lacticin were present in the appropriate cheeses implying that bacteriocin integrity was  
534 not compromised in the cheese environment. The presence of plantaricin could not be  
535 confirmed in Vat 7 cheese (double producer and plantaricin) at week 4 but it was  
536 present in vats 5 and 6 at both times as expected. MALDI-TOF MS is not quantitative  
537 and is also subject to preferential ionisation in that some peptides ionise better than  
538 others. The peptide content in a cheese increases during ripening due to the breakdown  
539 of casein so a number of bacteriocin purification steps were performed to increase the  
540 chances of detecting bacteriocin masses. Cheeses were passed through C18 SPE  
541 columns and peptides were further separated using RP-HPLC. Each HPLC fraction  
542 potentially contains numerous peptides making it difficult to detect the bacteriocin  
543 masses which are present at low concentrations. Usually the bacteriocin mass and a  
544 concomitant zone of inhibition is taken as proof of the presence of bacteriocin but in the  
545 case of a cheese fraction the presence of a zone of inhibition alone may be taken as  
546 indicative of bacteriocin presence.

547 Natural isolates capable of producing multiple bacteriocins have been reported  
548 in the literature (47-51). Most recently, *L. lactis* LMG2081 was shown to produce two  
549 different Classes of bacteriocins, a novel lantibiotic and the Class IIb bacteriocin,

550 lactococcin G (52). However, the ability to generate a multi-bacteriocin producer from  
551 an already established culture through food-grade enabling technologies poses several  
552 benefits. Firstly, the technological properties of the culture are known. The number of  
553 cultures required to produce multiple bacteriocins is reduced. The risk of bacteriocin  
554 inhibition is removed since the multibacteriocin-producing starter will also harbour the  
555 genetic machinery for bacteriocin immunity. As conjugation is a natural process, the  
556 resulting transconjugants do not fall under current European regulations governing the  
557 use of genetically modified microorganisms (53, 54). Therefore, transconjugants can be  
558 used in food applications in a similar manner to the recipient strain (55). While the  
559 double bacteriocin producer generated in this study proved to be a slower acidifier than  
560 the recipient strain, it has potential to serve as a protective culture. However, studies  
561 generating multiple bacteriocin producers have been rare (13). This can most likely be  
562 attributed to the complex biosynthetic process required for bacteriocin production and  
563 secretion. Indeed, previous attempts to construct nisin-lacticin transconjugants were  
564 unsuccessful, often attributed to the incompatibility of bacteriocin modification  
565 machinery or bacteriocin sensitivity (13, 30). Traditionally, the discovery of  
566 technologically valuable industrial strains has focused on large-scale screening  
567 strategies from a variety of sources (56). However, the successful transfer of lacticin  
568 and nisin to commercial starter cultures as reported in this study may provide additional  
569 avenues for the development of multi-hurdle protective cultures using food-grade  
570 methods.

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## REFERENCES

1. Mills S, O' Sullivan O, Hill C, Fitzgerald GF, Ross RP. 2010. The changing face of dairy starter culture research: from genomics to economics. *Int J Dairy Technol* **63**:149-170.
2. O' Sullivan L, Morgan SM, Ross RP. 2007. Bacteriocins: changes in cheese flora and flavour, p 326-348. *In* Weimer BC (ed), *Improving the flavour of cheese*, Woodhead Publishing, Cambridge, England.
3. O' Connor PM, Ross RP, Hill C, Cotter PD. 2015. Antimicrobial antagonists against food pathogens: a bacteriocin perspective. *Curr Opin Food Sci* **2**:51-57.
4. Cotter PD, Hill C, Ross RP. 2005. Bacteriocins: developing innate immunity for food. *Nat Rev Microbiol* **3**:777-788.
5. Alvarez-Sieiro P, Montalbán-López M, Mu D, Kuipers OP. 2016. Bacteriocins of lactic acid bacteria: extending the family. *Appl Microbiol Biotechnol* **100**: 2939–2951.
6. Martinez RC, Staliano CD, Vieira AD, Villarreal ML, Todorov SD, Saad SM, Franco BD. 2015. Bacteriocin production and inhibition of *Listeria monocytogenes* by *Lactobacillus sakei* subsp. *sakei* 2a in a potentially synbiotic cheese spread. *Food Microbiol* **48**:143-152.
7. Diaz-Ruiz G, Omar NB, Abriouel H, Canamero MM, Galvez A. 2012. Inhibition of *Listeria monocytogenes* and *Escherichia coli* by bacteriocin-producing *Lactobacillus plantarum* EC52 in a meat sausage model system. *African J Microbiol Res* **6**:1103-1108.
8. Garde S, Avila M, Arias R, Gaya P, Nunez M. 2011. Outgrowth inhibition of *Clostridium beijerinckii* spores by a bacteriocin-producing lactic culture in ovine milk cheese. *Int J Food Microbiol* **150**:59-65.
9. Gómez-Sala B, Herranz C, Díaz-Freitas B, Hernández PE, Sala A, Cintas LM. 2016. Strategies to increase the hygienic and economic value of fresh fish: Biopreservation using lactic acid bacteria of marine origin. *Int J Food Microbiol* **223**: 41-49.
10. Mills S, Stanton C, Hill C, Ross RP. 2011. New developments and applications of bacteriocins and peptides in foods. *Ann Rev Food Sci Technol* **2**:299-329.
11. Horn N, Martinez MI, Martinez JM, Hernandez PE, Gasson MJ, Rodriguez JM, Dodd HM. 1999. Enhanced production of pediocin PA-1 and coproduction of nisin and pediocin PA-1 by *Lactococcus lactis*. *Appl Environ Microbiol* **65**:4443-4450.
12. Reviriego C, Fernandez L, Rodriguez JM. 2007. A food-grade system for production of pediocin PA-1 in nisin-producing and non-nisin-producing

- 627 *Lactococcus lactis* strains: application to inhibit *Listeria* growth in a cheese  
628 model system. J Food Prot **70**:2512-2517.  
629
- 630 13. O'Sullivan L, Ryan MP, Ross RP, Hill C. 2003. Generation of food-grade  
631 lactococcal starters which produce the lantibiotics lacticin 3147 and lacticin 481.  
632 Appl Environ Microbiol **69**:3681-3685.  
633
- 634 14. Breukink E, Wiedemann I, van Kraaij C, Kuipers OP, Sahl HG, de Kruijff  
635 B. 1999. Use of the cell wall precursor lipid II by a pore-forming peptide  
636 antibiotic. Science **286**: 2361-2364.  
637
- 638 15. Lubelski J, Rink R, Khusainov R, Moll GN, Kuipers OP. 2008. Biosynthesis,  
639 immunity, regulation, mode of action and engineering of the model lantibiotic  
640 nisin. Cell Mol Life Sci **65**:455-476.  
641
- 642 16. Wiedemann I, Bottiger T, Bonelli RR, Wiese A, Hagge SO, Gutschmann T,  
643 Seydel U, Deegan L, Hill C, Ross P, Sahl HG. 2006. The mode of action of the  
644 lantibiotic lacticin 3147- a complex mechanism involving specific interaction of  
645 two peptides and the cell wall precursor lipid II. Mol Microbiol **61**:285-296.  
646
- 647 17. Suda S, Cotter PD, Hill C, Ross RP. 2012. Lacticin 3147 – biosynthesis,  
648 molecular analysis, immunity, bioengineering and applications. Curr Protein  
649 Pept Sci **13**:193-204.  
650
- 651 18. Mills S, Serrano LM, Griffin C, O'Connor PM, Schaad G, Bruining C, Hill  
652 C, Ross RP, Meijer WC. 2011. Inhibitory activity of *Lactobacillus plantarum*  
653 LMG P-26358 against *Listeria innocua* when used as an adjunct starter in the  
654 manufacture of cheese. Microb Cell Fact **10 Suppl 1**:S7.  
655
- 656 19. Cui Y, Zhang C, Wang Y, Shi J, Zhang L, Ding Z, Qu X, Cui H. 2012. Class  
657 IIa bacteriocins: diversity and new developments. Int J Mol Sci **13**:16668-  
658 16707.  
659
- 660 20. Van Tassell ML, Ibarra-Sánchez LA, Takhar SR, Amaya-Llano SL, Miller  
661 MJ. 2015. Use of a miniature laboratory fresh cheese model for investigating  
662 antimicrobial activities. J. Dairy Sci. 98: 8515-8524.  
663
- 664 21. Schelegueda LI, Delcarlo SB, Gliemmo MF, Campos CA. 2016. Effect of  
665 antimicrobial mixtures and modified atmosphere packaging on the quality of  
666 Argentine hake (*Merluccius hubbsi*) burgers. LWT Food Sci Technol **68**: 258-  
667 264.  
668
- 669 22. Olle Resa CP, Gerschenson LN, Jagus RJ. 2014. Natamycin and nisin  
670 supported on starch edible films for controlling mixed culture growth on model  
671 systems and Port Salut cheese. Food Control **44**: 146-151.  
672
- 673 23. Fernandez MV, Jagus RJ, Mugliaroli SL. 2014. Effect of combined natural  
674 antimicrobials on spoilage microorganisms and *Listeria innocua* in a whey  
675 cheese "Ricotta." Food Bioprocess Technol **7**: 2528-2537.  
676

24. **Mingming G, Jin TZ, Wang L, Scullen J, Sommers CH.** 2014. Antimicrobial films and coatings for inactivation of *Listeria innocua* on ready-to-eat deli turkey meat. *Food Control* **40**: 64-70.
25. **Morgan SM, Ross RP, Beresford T, Hill C.** 2000. Combination of hydrostatic pressure and lacticin 3147 causes increased killing of *Staphylococcus* and *Listeria*. *J Appl Microbiol* **88**: 414-420.
26. **Scannell AG, Ross RP, Hill C, Arendt K.** 2000. An effective lacticin biopreservative in fresh pork sausage. *J Food Prot* **63**: 370-375.
27. **Soriano A, Ulmer HM, Scannell AGM, Ross RP, Hill C, Garcia-Ruiz A, Arendt EK.** 2004. Control of food spoiling bacteria in cooked meat products with nisin, lacticin 3147, and a lacticin 3147-producing starter culture. *Eur Food Res Technol* **219**: 6-13.
28. **Scannell AG, Hill C, Ross RP, Marx S, Hartmeier W, Elke, Arendt K.** 2000. Development of bioactive food packaging materials using immobilised bacteriocins lacticin 3147 and nisaplin. *Int J Food Microbiol* **60**: 241-249.
29. **De Man JC, Rogosa M, Sharpe ME.** 1960. A medium for the cultivation of lactobacilli. *J Appl Microbiol* **23**: 130-135.
30. **Coakley M, Fitzgerald G, Ross RP.** 1997. Application and evaluation of the bacteriophage resistance- and bacteriocin-encoding plasmid pMRC01 for the improvement of dairy starter cultures. *Appl Environ Microbiol* **63**:1434-1440.
31. **Gireesh T, Davidson BE, Hillier AJ.** 1992. Conjugal transfer in *Lactococcus lactis* of a 68-kilobase-pair chromosomal fragment containing the structural gene for the peptide bacteriocin nisin. *Appl Environ Microbiol* **58**:1670-1676.
32. **Ryan MP, Rea MC, Hill C, Ross RP.** 1996. An application in Cheddar cheese manufacture for a strain of *Lactococcus lactis* producing a novel broad-spectrum bacteriocin, lacticin 3147. *Appl Environ Microbiol* **62**:612-619.
33. **Field D, Begley M, O'Connor PM, Daly KM, Hugenholtz F, Cotter PD, Hill C, Ross RP.** 2012. Bioengineered nisin A derivatives with enhanced activity against both Gram positive and Gram negative pathogens. *PLoS One* **7**:e46884.
34. **Hoffman CS, Winston F.** 1987. A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. *Gene* **57**:267-272.
35. **Mills S, Griffin C, Coffey A, Meijer WC, Hafkamp B, Ross RP.** 2010. CRISPR analysis of bacteriophage insensitive mutants (BIMs) of *Streptococcus thermophilus* - implications for starter design. *J Appl Microbiol* **108**:945-955.
36. **Mills S, Coffey A, McAuliffe OE, Meijer WC, Hafkamp B, Ross RP.** 2007. Efficient method for generation of bacteriophage insensitive mutants of

- 726 *Streptococcus thermophilus* yoghurt and mozzarella strains. J Microbiol  
727 Methods **70**:159-164.  
728
- 729 37. **Harrington A, Hill C.** 1991. Construction of a Bacteriophage-Resistant  
730 Derivative of *Lactococcus lactis* subsp. *lactis* 425A by Using the Conjugal  
731 Plasmid pNP40. Appl Environ Microbiol **57**:3405-3409.  
732
- 733 38. **Gravesen A, Jydegaard Axelsen AM, Mendes da Silva J, Hansen TB,**  
734 **Knochel S.** 2002. Frequency of bacteriocin resistance development and  
735 associated fitness costs in *Listeria monocytogenes*. Appl Environ Microbiol  
736 **68**:756-764.  
737
- 738 39. **Fallico V, McAuliffe O, Fitzgerald GF, Hill C, Ross RP.** 2009. The presence  
739 of pMRC01 promotes greater cell permeability and autolysis in lactococcal  
740 starter cultures. Int J Food Microbiol **133**:217-224.  
741
- 742 40. **Smith JS, Hillier AJ, Lees GJ.** 1975. The nature of the stimulation of the  
743 growth of *Streptococcus lactis* by yeast extract. J Dairy Res **42**: 123-138.  
744
- 745 41. **Friebs K.** 2004. Plasmid copy number and plasmid stability. Adv Biochem Eng  
746 Biotechnol **86**: 47-82.  
747
- 748 42. **Bravo D, Rodriguez E, Medina M.** 2009. Nisin and lacticin 481 coproduction  
749 by *Lactococcus lactis* strains isolated from raw ewes' milk. J Dairy Sci **92**:4805-  
750 4811.  
751
- 752 43. **Avonts, L., E. Van Uytven, and L. De Vuyst.** 2004. Cell growth and  
753 bacteriocin production of probiotic *Lactobacillus* strains in different media. Int.  
754 Dairy J. **14**: 947–955.  
755
- 756 44. **Bastos Mdo C, Coelho ML, Santos OC.** 2015. Resistance to bacteriocins  
757 produced by Gram-positive bacteria. Microbiology **161**: 683-700.  
758
- 759 45. **Donnelly CW.** 2004. Growth and survival of microbial pathogens in cheese, p  
760 541-560. In Fox PF, McSweeney PLH, Cogan TM, Guinee TP (ed), Cheese  
761 Chemistry, Physics and Microbiology 3<sup>rd</sup> Edition, Vol 1, Elsevier Academic  
762 Press, London.  
763
- 764 46. **Johnson EA, Nelson JH, Johnson M.** 1990. Microbiological safety of cheese  
765 made from heat-treated milk, Part II. Microbiology. J Food Prot **53**: 519-540.  
766
- 767 47. **O'Shea EF, O'Connor PM, Raftis EJ, O'Toole PW, Stanton C, Cotter PD,**  
768 **Ross RP, Hill C.** 2011. Production of multiple bacteriocins from a single locus  
769 by gastrointestinal strains of *Lactobacillus salivarius*. J Bacteriol **193**:6973-  
770 6982.  
771
- 772 48. **Quadri LE, Sailer M, Roy KL, Vederas JC, Stiles ME.** 1994. Chemical and  
773 genetic characterization of bacteriocins produced by *Carnobacterium piscicola*  
774 LV17B. J Biol Chem **269**:12204-12211.  
775

- 776 49. **Rodríguez E, González B, Gaya P, Nuñez M, Medina M.** 2000. Diversity of  
777 bacteriocins produced by lactic acid bacteria isolated from raw milk. *Int Dairy J*  
778 **10**:7-15.  
779
- 780 50. **Kojic M, Strahinic I, Fira D, Jovcic B, Topisirovic L.** 2006. Plasmid content  
781 and bacteriocin production by five strains of *Lactococcus lactis* isolated from  
782 semi-hard homemade cheese. *Can J Microbiol* **52**: 1110-1120.  
783
- 784 51. **Himeno K, Fujita K, Zendo T, Wilaipun P, Ishibashi N, Masuda Y,**  
785 **Yoneyama F, Leelawatcharamas V, Nakayama J, Sonomoto K.** 2012.  
786 Identification of enterocin NKR-5-3C, a novel Class IIa bacteriocin produced by  
787 a multiple bacteriocin producer, *Enterococcus faecium* NKR-5-3. *Biosci*  
788 *Biotechnol Biochem* **76**: 1245-1247.  
789
- 790 52. **Mirkovic N, Polovic N, Vukotic G, Jovcic B, Miljkovic M, Radulovic Z,**  
791 **Diep DB, Kojic M.** 2016. *Lactococcus lactis* LMG2081 produces two  
792 bacteriocins, a nonlantibiotic and a novel lantibiotic. *Appl Environ Microbiol.*  
793 **82**: 2555-2562.  
794
- 795 53. **Derkx PMF, Janzen T, Sørensen KI, Christensen JE, Stuer-Lauridsen B,**  
796 **Johansen E.** 2014. The art of strain improvement of industrial lactic acid  
797 bacteria without the use of recombinant DNA technology.  
798
- 799 54. **Pedersen MB, Iversen SL, Sørensen KI, Johansen E.** 2005. The long and  
800 winding road from the research laboratory to industrial applications of lactic  
801 acid bacteria. *FEMS Microbiol Rev* **29**: 611-624.  
802
- 803 55. **Hill C, Ross RP.** 1998. Starter cultures for the dairy industry, p 174-192. *In*  
804 *Roller S, Harlander S (ed), Genetic Modification in the Food Industry: A*  
805 *Strategy for Food Quality Improvement*, Springer Science, Dordrecht.  
806
- 807 56. **Hansen EB.** 2002. Commercial bacterial starter cultures for fermented foods of  
808 the future. *Int J Food Microbiol* **78**:119-131.  
809  
810  
811  
812  
813  
814  
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826 FIG 1 PCR amplification using pMRC01-specific primers (*orf27*, *orf49*, *orf51*, and  
827 *orf52*) to detect the presence of pMRC01 in *L. lactis* CSK3594 and CSK3533. PCR  
828 amplification using primers designed to regions of the nisin operon (*nisA*, *nisFEG*) to  
829 confirm the presence of nisin genetic determinants in *L. lactis* CSK3281 and *L. lactis*  
830 CSK3533. (M: 100 bp DNA ladder; New England BioLabs).

831 FIG 2 Colony mass spectrometry analysis of *L. lactis* CSK2775, *L. lactis* CSK3594  
832 (lactacin transconjugant, Ltn+), *L. lactis* CSK3281 (nisin transconjugant, Nis+) and *L.*  
833 *lactis* CSK3533 (nisin and lactacin double producer, Ltn+, Nis+). Masses corresponding  
834 to the bacteriocins are indicated. Inset photos show inhibition zones produced by each  
835 strain against the indicator strain *L. lactis* HP.

836 FIG 3 Acidification profiles of *L. lactis* CSK2775 (□); *L. lactis* CSK3594 (lactacin)  
837 (■); *L. lactis* CSK3281 (nisin) (○); and *L. lactis* CSK3533 (nisin, lactacin) (●) grown  
838 in 10% RSM.

839 FIG 4 Counts of viable *L. innocua* cells in laboratory-scale cheeses. Bacteriocin-  
840 containing vats were compared to Vat 1 (no bacteriocin) at each week (\* P<0.05; \*\*  
841 P<0.01; \*\*\* P<0.001).

842 FIG 5 MALDI-TOF MS analysis of Vat 7 (lactacin, nisin and plantaricin) (A), Vat 4  
843 (lactacin and nisin) (B), Vat 5 (nisin and plantaricin) (C) and Vat 6 (lactacin and  
844 plantaricin) (D). Masses corresponding to the bacteriocins are indicated. Inset photos  
845 show inhibition zones produced by correct mass-containing fractions against the  
846 indicator strains *L. lactis* HP (nisin and lactacin) or *L. innocua* (plantaricin) where F  
847 denotes Fraction.

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854 TABLE 1 Bacterial strains used in this study

Bacterial strain	Relevant Detail	Relevant genotype and phenotype <sup>a</sup>	Source or reference
<i>L. lactis</i> HP	Bacteriocin sensitive indicator strain	<i>Ltn</i> <sup>-</sup> ; <i>Nis</i> <sup>-</sup>	TFRC <sup>b</sup>
<i>L. lactis</i> MG1363 (pMRC01)	Donor strain harbouring pMRC01, lacticin producer	<i>Lac</i> <sup>-</sup> ; <i>Ltn</i> <sup>+</sup> ; <i>Nis</i> <sup>-</sup>	TFRC
<i>L. lactis</i> CSK2583	Donor strain harbouring Tn5276, nisin producer	<i>Lac</i> <sup>-</sup> ; <i>Nis</i> <sup>+</sup> ; <i>Ltn</i> <sup>-</sup>	CSK, The Netherlands
<i>L. lactis</i> CSK2775	Recipient strain	<i>Lac</i> <sup>+</sup> ; <i>Nis</i> <sup>-</sup> ; <i>Ltn</i> <sup>-</sup>	CSK, The Netherlands
<i>L. lactis</i> CSK3281	CSK2775 derivative, nisin producer	<i>Lac</i> <sup>+</sup> ; <i>Nis</i> <sup>+</sup>	This study
<i>L. lactis</i> CSK3594	CSK2775 transconjugant harbouring pMRC01, lacticin producer	<i>Lac</i> <sup>+</sup> ; <i>Ltn</i> <sup>+</sup>	This study
<i>L. lactis</i> CSK3533	CSK3281 transconjugant harbouring pMRC01, nisin-lacticin double producer	<i>Lac</i> <sup>+</sup> ; <i>Nis</i> <sup>+</sup> ; <i>Ltn</i> <sup>+</sup>	This study
<i>Lb. plantarum</i> LMG P-2658	Plantaricin 423 producer	<i>Pln</i> <sup>+</sup>	(18)
<i>L. lactis</i> DPC4268	Starter culture for cheese manufacture	<i>Lac</i> <sup>+</sup>	TFRC

855 <sup>a</sup>*Lac*, lactose utilization; *Ltn*, lacticin genetic determinants; *Nis*, nisin genetic determinants; *Pln*,  
856 plantaricin genetic determinants.

857 <sup>b</sup>TFRC, Teagasc Food Research Centre, Moorepark, Fermoy, County Cork, Ireland.

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874 TABLE 2 Antimicrobial spectrum of the *L. lactis* isogenic family of nisin and lacticin transconjugants

Indicator Strain or Species	Growth Medium	Strains Tested for Antimicrobial Activity				Source
		CSK2275	CSK3594 (Ltn)	CSK3281 (Nis)	CSK3533 (Ltn, Nis)	
<i>Bacillus cereus</i> DPC6085/6086	BHI <sup>a</sup>	No zone	No zone	1mm zone	1mm zone	TFRC
<i>Bacillus subtilis</i> DPC6511	BHI <sup>a</sup>	No zone	No zone	No zone	No zone	TFRC
<i>Enterococcus faecalis</i> DPC5055/LMG 7973	BHI <sup>a</sup>	No zone	No zone	3mm zone	3mm zone	TFRC
<i>Enterococcus faecium</i> DPC5056 <sup>c</sup>	BHI <sup>a</sup>	No zone	No zone	6mm zone	6mm zone	TFRC
<i>Escherichia coli</i> P1432- DPC6054	BHI <sup>a</sup>	No zone	No zone	No zone	No zone	TFRC
<i>Clostridium sporogenes</i> DPC6341 <sup>a</sup>	RCM <sup>a</sup>	No zone	1.5 mm zone	1.5mm zone	1mm zone	TFRC
<i>Clostridium tyrobutyricum</i> DPC6342 <sup>a</sup>	RCM <sup>a</sup>	1mm zone	4mm zone	3mm zone	6mm zone	TFRC
<i>Lactobacillus casei</i> DPC6125	MRS <sup>c</sup>	No zone	No zone	7mm zone	7mm zone	TFRC
<i>Lactobacillus acidophilus</i> DPC5378	MRS <sup>a</sup>	No zone	1mm zone	3mm zone	3mm zone	TFRC
<i>Lactobacillus delbreukii</i> subsp. <i>delbreukii</i> DPC5385	MRS <sup>a</sup>	No zone	No zone	6mm zone	6mm zone	TFRC
<i>Lb. delbreukii</i> subsp. <i>lactis</i> DPC5387	MRS <sup>c</sup>	No zone	1.5mm zone	6mm zone	6mm zone	TFRC
<i>Lb. delbreukii</i> subsp. <i>bulgaricus</i> DPC5383	MRS <sup>c</sup>	No zone	2mm zone	9.5mm zone	9.5mm zone	TFRC
<i>Lactobacillus helveticus</i> DPC4571	MRS <sup>a</sup>	No zone	1.5mm zone	8mm zone	8mm zone	TFRC
<i>L. lactis</i> subsp. <i>lactis</i> biovar <i>diacetylactis</i> CSK1411	LM17 <sup>b</sup>	No zone	1.5mm zone	4mm zone	4mm zone	CSK
<i>L. lactis</i> subsp. <i>cremoris</i> HP DPC5718	LM17 <sup>c</sup>	No zone	2.5mm zone	4.5mm zone	4.5mm zone	TFRC
<i>L. lactis</i> subsp. <i>lactis</i> DPC4268/ 303	LM17 <sup>c</sup>	No zone	No zone	0.5mm zone	0.25mm zone	TFRC
<i>L. lactis</i> subsp. <i>lactis</i> CSK2775	LM17 <sup>c</sup>	No zone	2.5 mm	4.5 mm	4.5 mm	CSK
<i>L. lactis</i> subsp. <i>lactis</i> CSK3594	LM17 <sup>c</sup>	No zone	No zone	4.5 mm	4.5 mm	CSK
<i>L. lactis</i> subsp. <i>lactis</i> CSK3281	LM17 <sup>c</sup>	No zone	2.5 mm	No zone	4 mm	CSK
<i>L. lactis</i> subsp. <i>lactis</i> CSK3533	LM17 <sup>c</sup>	No zone	No zone	No zone	No zone	CSK
<i>Leuconoctos lactis</i> DPC3838	MRS <sup>c</sup>	No zone	No zone	1.5mm zone	1.5mm zone	TFRC
<i>L. innocua</i> DPC6578	GM17 <sup>a</sup>	No zone	No zone	No zone	No zone	TFRC

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876 \* Cultures grown anaerobically for up to 48h. <sup>a</sup>Cultures grown at 37°C for up to 48h. <sup>b</sup>Cultures grown at 35°C for up to 48h. <sup>c</sup>Cultures  
877 grown at 30°C for up to 48h.  
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885 TABLE 3 Primer pairs used in this study

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Primer	Sequence	Target gene(s)	Size (bp)
27-F 5'-GGGGAACAATCTTACCTA	27-R 5'-ATTATTTTATTGCATTCTACTA	<i>orf27</i>	326
49-F 5'-CCAATACCCGCCAAAATAAAGT	49-R 5'-CTAAGCGCAGAGGAAATACAACC	<i>orf49</i>	347
51-F 5'-TTCTCAAAATCATCAAAATCAAGT	51-R 5'-GTACGAACAGGAGCGAAAAA	<i>orf51</i>	293
52-F 5'- CCTAAGTTGTCTATTCGTGTCCA	52-R 5'- ATTAGGTGAGTGCTCTGATTTTC	<i>orf52</i>	210
nisA-F 5'- CAAAAGATTTTAACTTGGATTG	nisA-R 5'- ACGTGAATACTACAATGACAAG	<i>nisA</i>	163
nisFG-F 5'- GGTTTAATTTCTGCAGATACTG	nisFG-R 5'- GTAATTATCCAGATCATTGCTG	<i>nisFEG</i>	1573

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904 TABLE 4 *L. lactis* transconjugants surveyed for bacteriophage sensitivity

Bacteriophage	<i>L. lactis</i> CSK2775	<i>L. lactis</i> CSK3281 (Nis <sup>+</sup> )	<i>L. lactis</i> CSK3594 (Ltn <sup>+</sup> )	<i>L. lactis</i> CSK3533 (Nis <sup>+</sup> Ltn <sup>+</sup> )
5410F	+	+	-	-
5163F	+	-	-	-
5210 F	+	+	+	+
5167F	+	-	-	-
5385F (Bacteriophage cocktail)	+	-	-	-
5386F (Bacteriophage cocktail)	+	+	-	-

905 + indicates bacteriophage sensitivity observed by a clearing of the bacterial population.

906 - indicates bacteriophage insensitivity observed as growth (turbidity) of the bacterial population.

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FIG 1

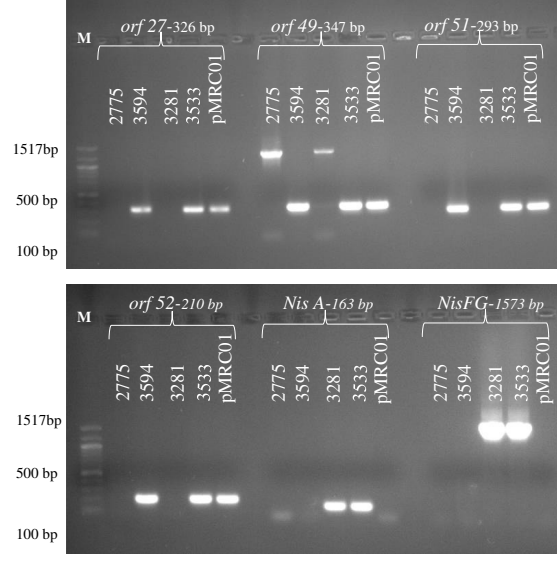


FIG 2

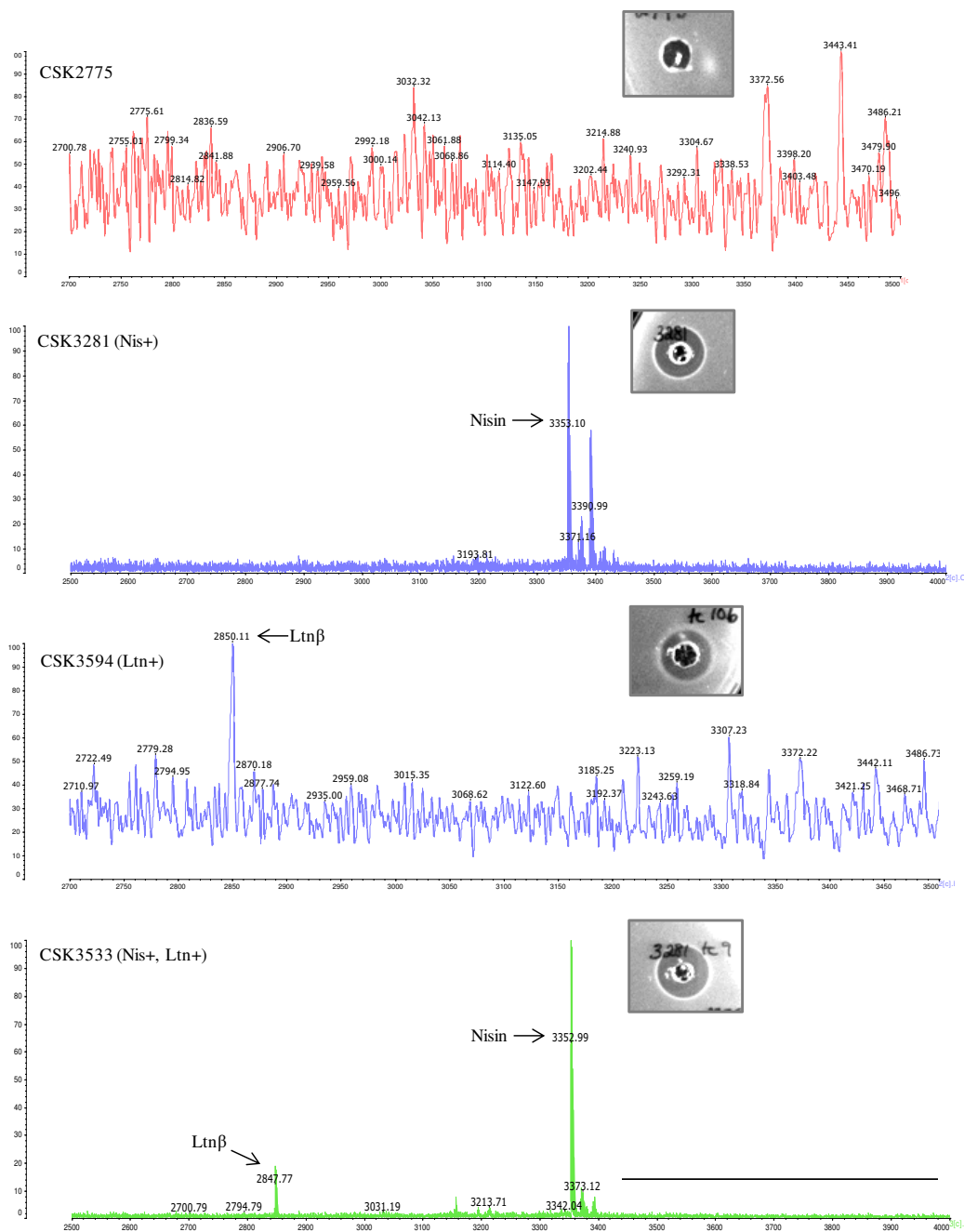


FIG 3

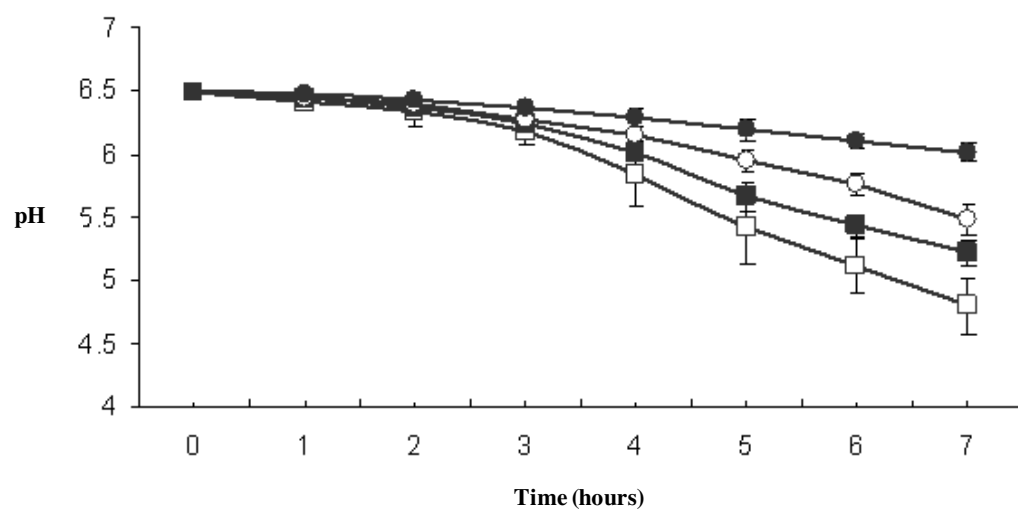


FIG 4

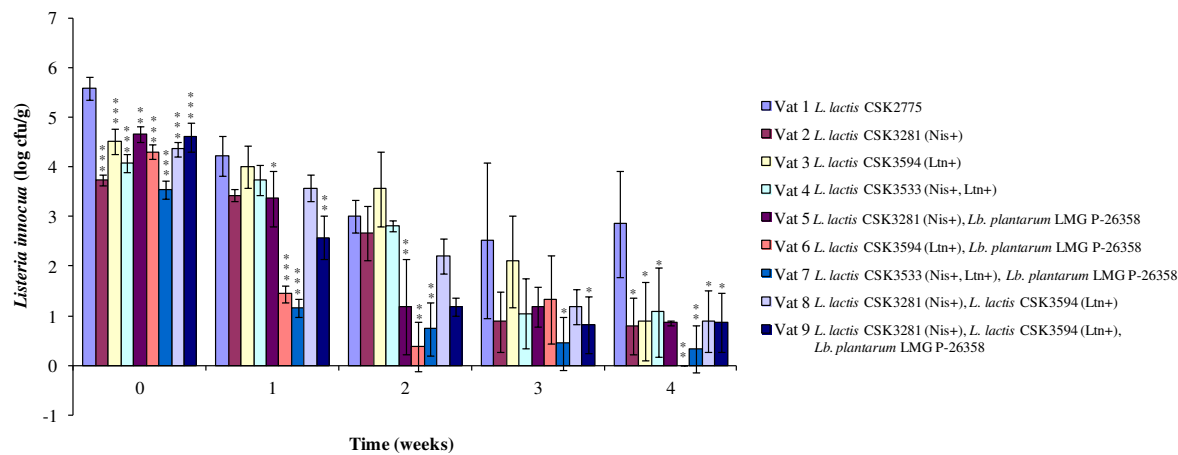


FIG 5 (A)

Vat 7 *L. lactis* CSK3533, *Lb. plantarum* LMG P-26358

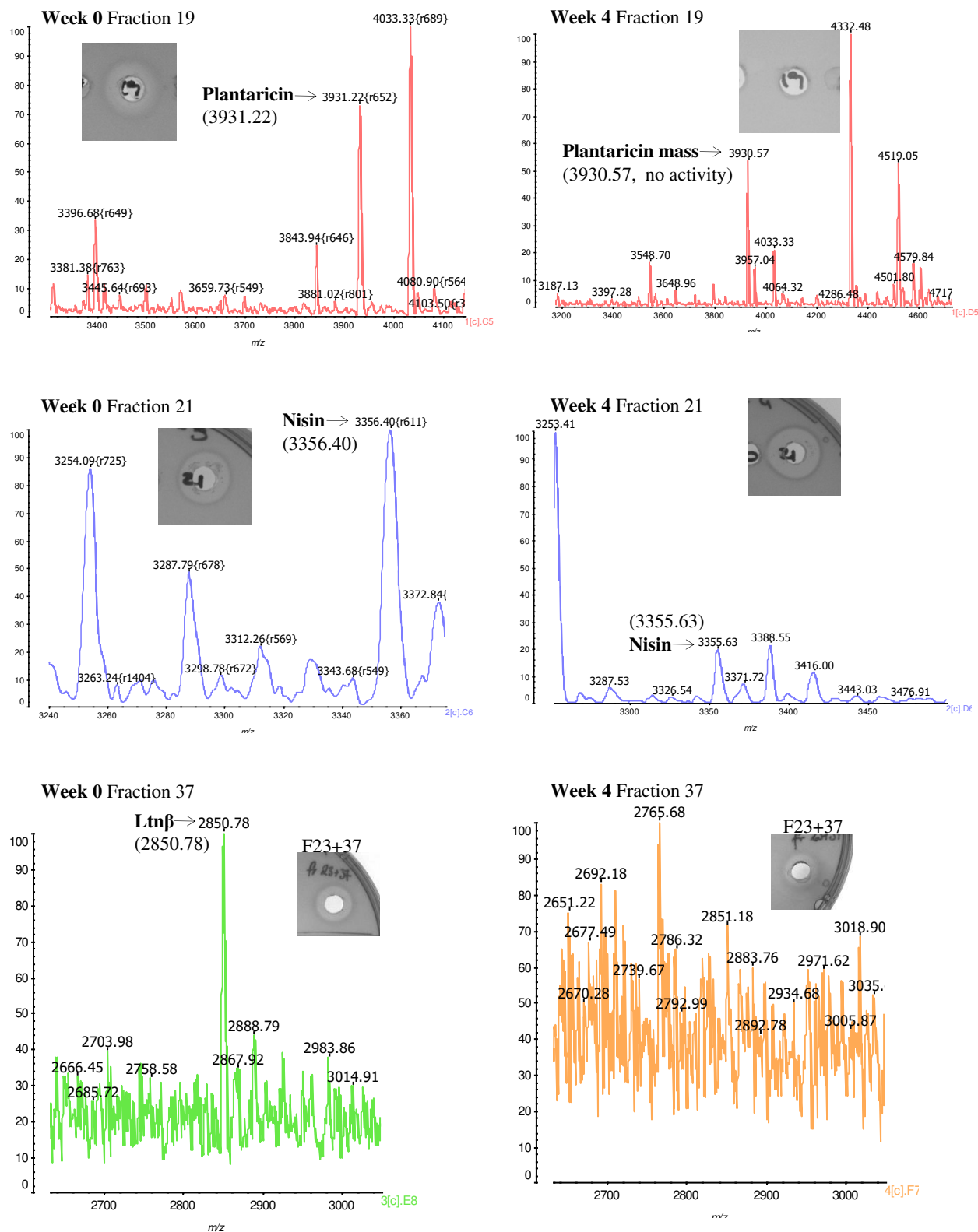


FIG 5 (B)

Vat 4 *L. lactis* CSK3533

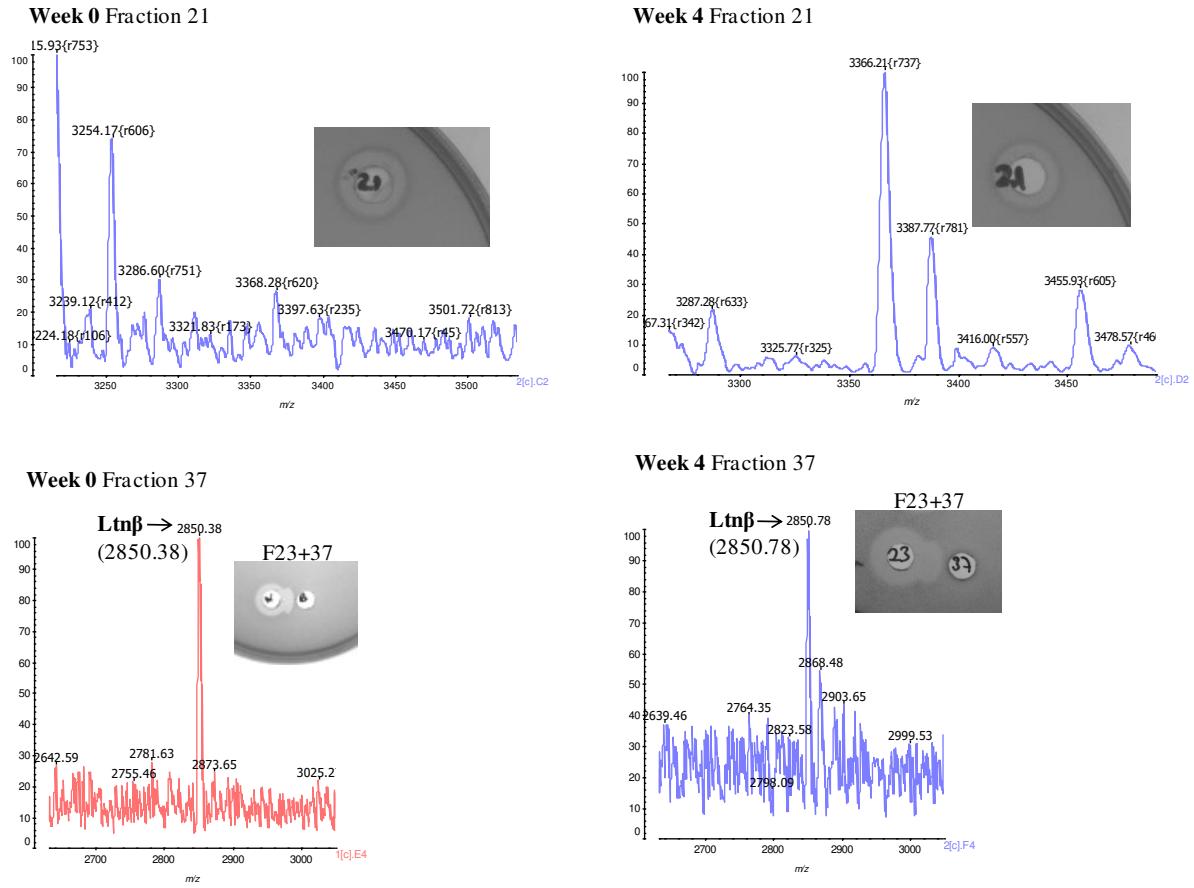




FIG 5 (C)

Vat 5 *L. lactis* CSK3281, *Lb. plantarum* LMG P-26358

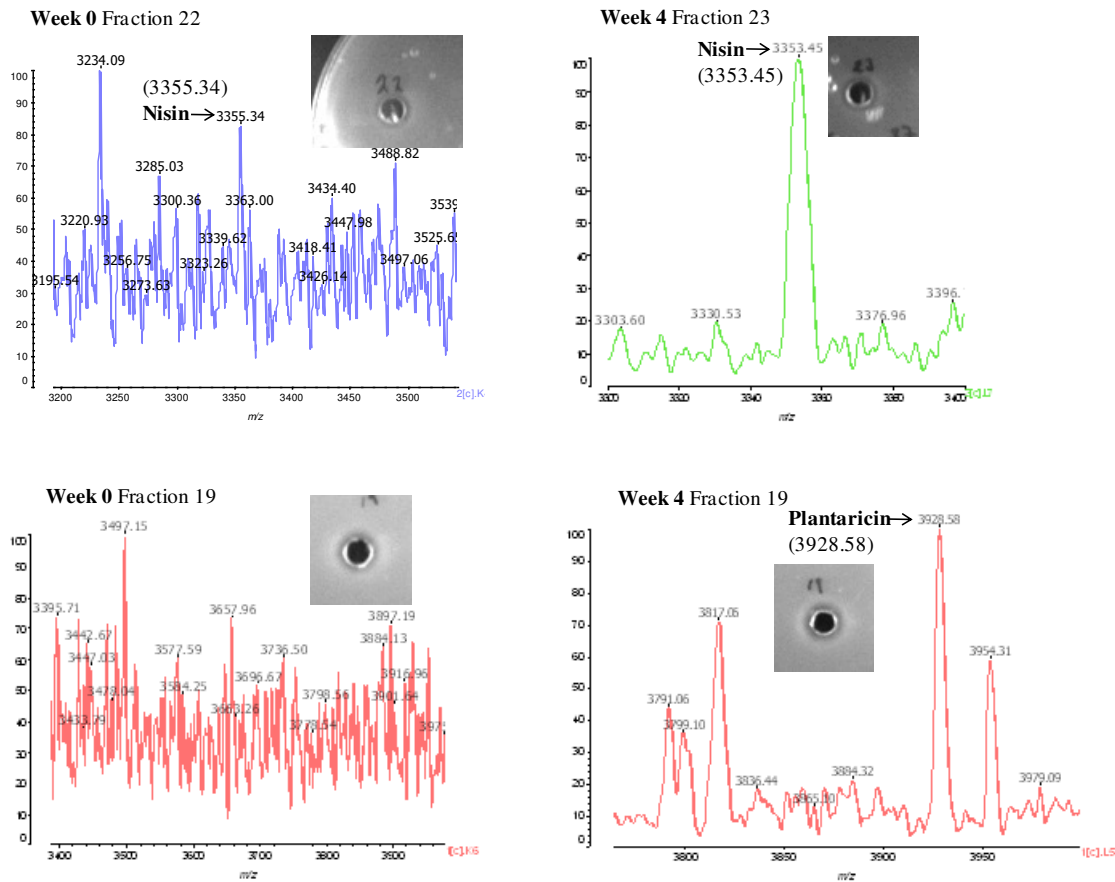
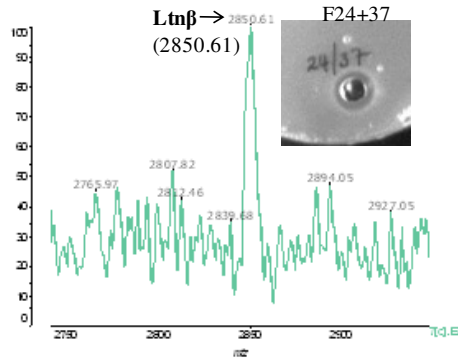


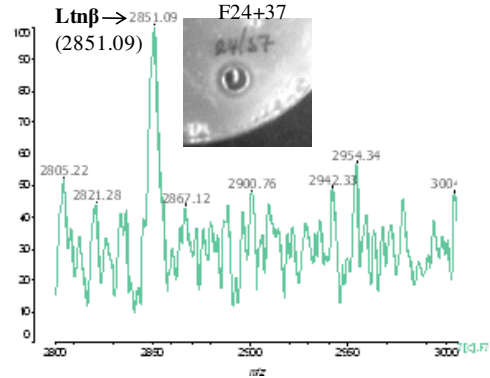
FIG 5 (D)

**Vat 6 *L. lactis* CSK3594, *Lb. plantarum* LMG P-26358**

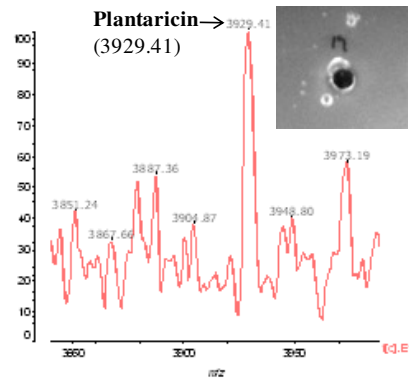
**Week 0 Fraction 37**



**Week 4 Fraction 37**



**Week 0 Fraction 19**



**Week 4 Fraction 19**

